THE TOXICOLOGY OF *Habrobracon* VENOM: A Study of a Natural Insecticide

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THE TOXICOLOGY OF *Habrobracon* VENOM: A Study of a Natural Insecticide[1]

Raimon L. Beard[2]

INTRODUCTION

In the valleys of the Orinoco and Amazon, the South American Indians have long known of roots and bark which when brewed yielded a gummy mass containing remarkable poisons. Arrow tips, dipped in such a concoction, brought prompt paralysis to victims of the Indian's marksmanship. The prey could be eaten safely, because the poison is not potent when taken in the alimentary tract. What once were tribal secrets are now known, and the arrow poisons, generally known as the curarine alkaloids, have become important pharmacologic drugs, if not very useful therapeutic agents (Gill, 1940).

Long before the South American Indians learned of this way of improving their hunting efficiency, a natural counterpart of the poisoned-arrow existed in certain wasps. These insects possessed built-in arrows and an apparatus for poisoning their tips. To be sure, these structures were used more like a hypodermic needle and syringe than an arrow shot from a blow-gun, but the end results are remarkably parallel. The wasps that use such an apparatus for paralyzing their prey are to be found among a number of families of Hymenoptera, and although their habits may vary some in details, the principal features of the phenomenon are quite similar. The natural history of these wasps has received wide attention, but the toxicology and pharmacology of their venoms have been almost completely neglected.

One of the many wasps that paralyze their prey by stinging is the principal subject of this report. Although to the entomologist this insect may be known as *Microbracon hebetor* Say, it is best known as *Habrobracon juglandis* Ashmead. This is one of the vagaries of insect taxonomy. Inasmuch as most of the work on this insect has been done under the name *Habrobracon* to the extent that a field of "habrobraconology" exists, and Martin (1947) has justified this name, it seems most useful to refer to the insect as *Habrobracon*.

The ease of rearing and the short life cycle have made *Habrobracon* a favorite object for genetic study, probably rating second only to *Drosophila* among insects in this respect. To Doctors A. R. Whiting and P. W. Whiting goes the credit for contributing most to habrobraconology, and a large part of the work done on this insect has been reviewed in book form by Martin

[1] The assistance of Miss Patricia Hoy and Mrs. M. B. Gentry, Jr., is gratefully acknowledged.
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(1947). The present writer is indebted to Dr. A. R. Whiting for providing stock cultures of wild strains of *Habrobracon* used in these studies and for suggestions as to rearing techniques.

Habrobracon BIOLOGY

The elements of *Habrobracon* biology have been described in the papers reviewed by Martin (1947).

Habrobracon[1] is a braconid wasp which requires for its development host larvae of such insects as *Ephestia, Plodia,* or *Galleria.*[2] It is said to be an ecto-parasite, but the female wasp is truly predaceous as are the larval stages. The female wasp paralyzes its host by injecting venom through its stinging apparatus. It feeds upon the blood which oozes from the wound made in the host integument. It has not been established whether or not additional venom is injected into host larvae with each feeding puncture made subsequent to the initial paralyzing sting. Oviposition is independent

Figure 1. *Habrobracon juglandis* Ashmead. Female. 14 x.

of the stinging and feeding behavior, the eggs being deposited on or very near to the paralyzed larvae. The male wasp does not feed on the host's blood, but will feed upon diluted honey if available (Doten, 1911). After leaving the egg, the wasp larvae cling to the host, and they, too, feed upon its blood by puncturing the integument (Figure 2). The host frequently shows target-shaped areas on its integument where the larva has made intimate contact during the feeding process. The wasp larva is not restricted to the particular host larva on or near which its egg was deposited, but if its food becomes depleted, or for other reasons, it may shift to another stung larva for further feeding. After completing larval development, the wasp larvae tend to leave the host and spin cocoons within which they pupate, later emerging as adults. As with many other Hymenoptera, females develop from fertilized eggs, whereas males develop from unfertilized eggs.

[1] Unless otherwise indicated, the species of *Habrobracon* referred to is *juglandis.*
[2] In most of the work here reported *Galleria* was the host used. Its larger size facilitated physiological study. *Ephestia,* however, was used to supplement host material in rearing the wasps.

Bender (1943) has described the stinging apparatus (Figure 3). The poison glands consist of (usually) eight elongated lobes, made up of a single layer of cells surrounding a central lumen, which communicate to the base of a central ovate reservoir. The poison reservoir is surrounded by a

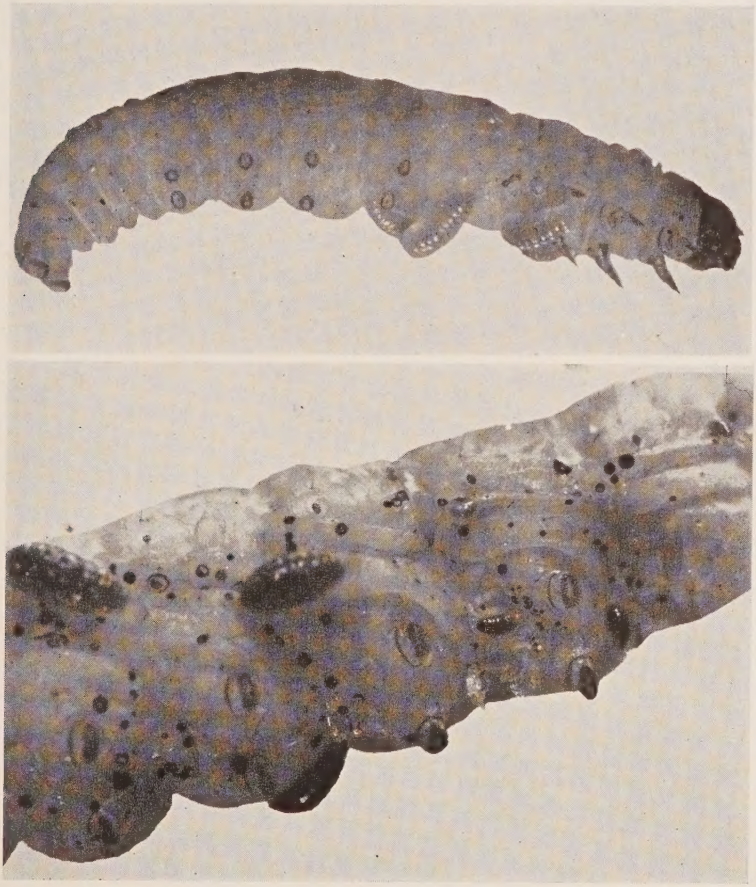

Figure 2. Upper photograph, *Habrobracon* larvae feeding on *Ephestia* larva. Note color similarity between predator and host. Lower photograph, *Habrobracon* larvae feeding on *Galleria* larva. Note dark appearance of predator and dark areas on host where wasp larvae have fed. This melanization reflects the rich tyrosine-tyrosinase system in *Galleria* blood, which is negligible in *Ephestia*. 8 x.

heavy muscular coat and lined by a characteristic cuticular spiral. A poison duct, surrounded by glandular cells, leads from the reservoir to the poison canal which is formed by the longitudinal space between the first and second valvulae. These valvulae constitute a dart-like structure—the sting. As a wasp stings its host, the first, slightly barbed, valvulae dart back and forth,

riding on the "rails" formed by the fused second valvulae, and thus work their way through the body wall of the host larva.

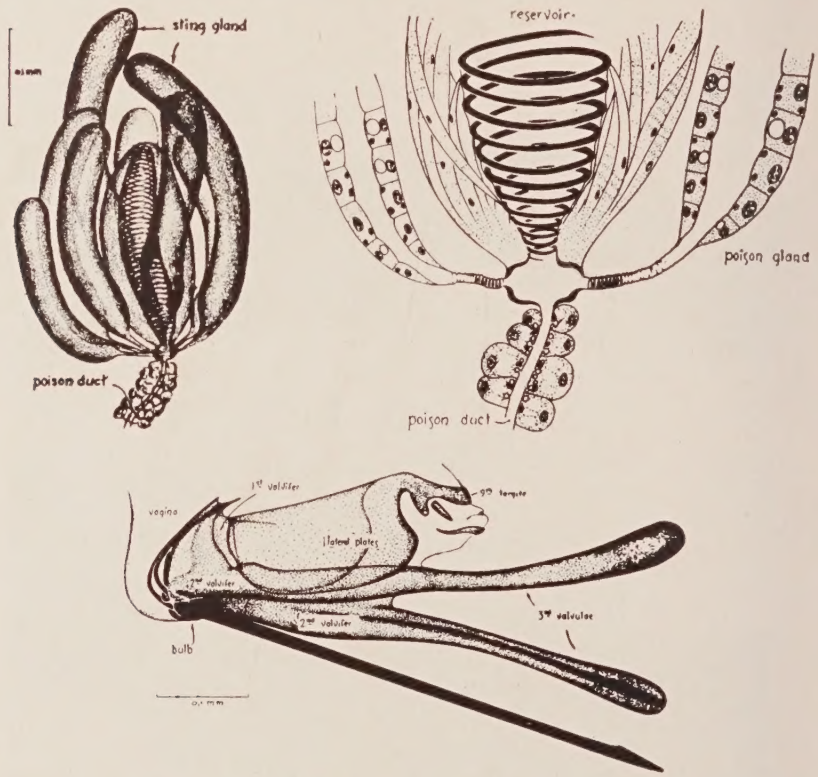

Figure 3. Upper left, poison glands and poison reservoir of *Habrobracon juglandis*. Upper right, longitudinal section of poison glands and reservoir with muscular coat. Lower sketch, ventro-lateral view of the ovipositor and associated parts. After Bender, 1943.

REARING METHODS

For genetic studies, the rearing methods outlined by Martin are appropriate. For mass rearings, in which individual matings are not necessary, a somewhat simpler technique is feasible. For the studies here reported, rearing was done in four by four-inch plastic refrigerator boxes. Into each box are placed from 50 to 80 host larvae (*Galleria, Ephestia,* or both) and from 10 to 15 pairs of *Habrobracon* adults. Initially, a solid cover is placed on the box, but after the host larvae are stung, this cover is replaced with cloth to allow circulation of air, thus reducing the possibility of mold. Greatest yield of offspring results with a ratio of about five host larvae per female wasp. Greater numbers of host larvae sometimes result in some escaping from being stung. Greater numbers of wasps result in too great a competi-

tion for food. Competition between the adult wasps and their larval offspring is reduced somewhat by removing the adult wasps two to three days after their introduction to the host larvae. Sufficient eggs are deposited during this time to insure good production, and removal of the wasps leaves the food for the exclusive use of the developing young. In view of the fact that the females oviposit over a period of several weeks, this method of rearing fails to utilize the full reproductive capacity of the wasps unless they are transferred to other hosts—which may be done.

Martin (1947) cites the optimum temperature for development as being 29-30° C. with a relative humidity of 80 per cent, although the life cycle is completed in the shortest time at 33.5° C. and a relative humidity of 32-98 per cent. At optimum conditions the life cycle is stated to require about two days for the egg, two days for the larva, and about four days for the pupa.

PARALYSIS OF THE HOST

The paralysis induced by the sting of the wasp is a striking phenomenon —not only as manifested by an individual affected by the venom, but as manifested by a group of *Galleria* larvae exposed to wasps.

Figure 4. *Galleria* larvae paralyzed by *Habrobracon*, showing stages of degeneration. Fecal discharge indicates continued gut activity in spite of paralysis. 3 x.

Symptoms of poisoning are rapidly manifested in an individual larva after it has been stung. At the time of stinging, the larva shows some initial signs of irritation, frequently biting at the wasp or at the point of stinging. As the stung larva crawls along, its normal progress shortly comes to a halt. Attempted locomotor activity continues for a fraction of a minute, but no progress is made—the insect seems to walk in one spot. As purposive movement ceases, there is a gradual loss of irritability, and the larva soon fails to respond to tactile stimulation. Also, externally applied electrical stimuli are without effect at this stage. For a longer or shorter time there are localized, irregular, and uncoordinated muscular fibrillations which gradually diminish as the flaccid paralysis becomes complete. The mouth parts are among the last structures to stop movement, with the exception of the heart and the gut. These latter internal structures appear unaffected by the poison. Heart action appears perfectly normal in a paralyzed larva, but after several days it gradually slows down, presumably because of the

Figure 5. Large group of *Galleria* larvae, each of which has been paralyzed by *Habrobracon*.

general debility of the insect. Gut activity, too, continues, but inasmuch as feeding ceases and other physiological features change, it is doubtful if the gut activity can be considered "normal". Certainly gut movement does not cease, and discharge of accumulated fecal material rather characteristically occurs within two to three days after the onset of paralysis. If death occurs in the early days of paralysis, the larva rapidly decomposes, presumably as a result of autolytic enzymes and the growth of putrefactive bacteria. If death and such post-mortem changes do not occur early, the larvae more

characteristically become more and more contracted and desiccated, particularly if *Habrobracon* feeding is extensive, until finally the victim is shrunken and dried (Figure 4).

Also impressive is the gross effect of paralysis when a large group of *Galleria* larvae are vulnerable to attack by *Habrobracon* (Figure 5). Although with an insufficient number of *Habrobracon* or given suitable hiding places, individuals in a group of *Galleria* may escape being stung, the efficiency and thoroughness of *Habrobracon* attack are surprisingly great.

ANTIBIOSIS

Although the whole stinging phenomenon is encompassed by Burkholder's (1952) definition of antibiosis as "a special case of antagonism in which an organism produces an injurious compound which may inhibit growth or destroy another organism", it does not fall within the usual concept of this term. Nevertheless, it has been popularly assumed that the venom of parasitic (predaceous) wasps is an antibiotic (antiseptic) in the sense that when injected into host insects it prevents growth of, or destroys, pathogenic microorganisms, thus assuring uncontaminated food for the wasps' offspring. The writer has seen no evidence that venom has been tested experimentally as an antibiotic. It may be that absence of microbial decomposition in paralyzed larvae has been interpreted as an indication of antibiotic activity. In fact, however, this may only mean that until death occurs and tissues decompose, the host's natural resistance to microbial invasion continues to operate, and there is not likely to be any source of inoculation. Certainly in *Galleria* larvae the presence of *Habrobracon* venom is no assurance that the larvae will remain uncontaminated. *Ephestia* larvae usually, but not always, remain free from disease and merely become desiccated when the wasp larvae complete their feeding. *Galleria* larvae, on the other hand, not infrequently decompose soon after being stung. Putrefactive bacteria are evident and the body turns black. On occasion so many larvae are thus affected that the disease assumes epizootic proportions, and the culture of *Habrobracon* is threatened. Thus, instead of its venom acting as an antibiotic, the wasp becomes suspect as a vector in the transmission of disease by inoculating a healthy larva after having stung a contaminated larva. Early symptoms of disease are usually absent, particularly in paralyzed larvae, so it is very difficult to judge whether discoloration and associated pathological conditions are manifestations of the causative agent or are the consequence of death from some other cause. Presumably both cause and effect are represented. In those cases of isolated, infrequent, premature deaths in an otherwise satisfactory culture, the melanization and bacterial decomposition presumably follow death. On the other hand, the situations in which an epizootic develops probably represent causal conditions, and the wasp is the most likely means of transmitting the disease from one host to another.

These observations recall the report by Payne (1933) that *Habrobracon* (*Microbracon*) served to transmit a sporozoan disease in *Ephestia* caused by *Thelohania ephestiae* Mattes. She inferred ganglionic stinging and noted that the initial infection centered in a ganglion—presumably at the point of stinging.

LOCUS OF STINGING

To some wasps of this type has been attributed the ability to insert the sting into a ganglion of the victim, thus introducing the venom directly into the nervous system. As mentioned above, this was inferred by Payne (1933) for *Habrobracon* stinging *Ephestia*. Some observers have seen in this concept a prescience and a perception of host morphology on the part of the wasp not acceptable to other observers, and some have noted that repeated stings are made and assume that by virtue of the position assumed by the wasp as it stings, some chance puncture reaches a ganglion. As will be seen, if direct ganglionic poisoning is required in those cases reported, an entirely different type of action is indicated than in the phenomenon under discussion here. With prey of *Habrobracon*, ganglionic stinging is neither likely nor necessary. *Habrobracon* assumes no characteristic position

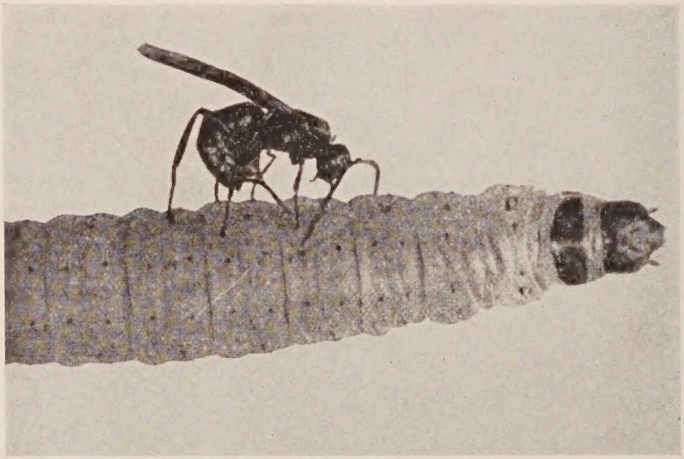

Figure 6. *Habrobracon* female stinging *Ephestia* larva. After Doten, 1911. Photograph courtesy of Dr. A. R. Whiting. 10 x.

relative to the host larvae when it stings, and apparently it is able to paralyze its prey by stinging at any locus (Figure 6). Effectiveness of the injected venom is assured by blood transport. That this occurs becomes evident from several observations. Extracts of poison glands injected parenterally into *Galleria* larvae result in paralysis. Moreover, blood taken from a paralyzed prey, when injected into the body cavities of other larvae, effectively paralyzes the latter. Blood transport is especially well demonstrated experimentally by ligating larvae and introducing venom parenterally on one side of the ligature. Paralysis sets in only on the side of the ligature where venom was injected, regardless of the location of the ligature. This indicates that no single central organ system needs to be inactivated, but tissues segmentally arranged are vulnerable. Blood transport becomes confirmed if the ligature is cut and flow is restored between both body regions. The unparalyzed portion of the larva then becomes paralyzed promptly.

EXPERIMENTAL "STINGING"

Blood transport and the potency of the venom make possible several methods of experimentally introducing the toxin into *Galleria* larvae. Of course, the larvae can be exposed to the wasps *ad libitum*, but it is also possible to hold a wasp against a larva in such a position as will encourage stinging. The small size of the wasp renders this somewhat difficult and not feasible for routine work, especially as the wasp, when restricted in its movements, is usually not attracted to the host larva. Its sting is then engaged in attempted defense rather than offense. Advantage can be taken of this, however, for when the stylets are extended, a minute droplet of venom is frequently ejected. This droplet can be collected on a needle or glass capillary and used, even though the droplet is so small that it dries almost immediately. This provides a "poisoned arrow" which can be inserted into the body cavity of a larva to induce paralysis. Such handling

Figure 7. "Poisoned arrow" technique of paralyzing larva. Upper, *Habrobracon* female held by suction capillary in position to apply venom, when ejected, to tip of needle. 13 x. Lower, *Galleria* larva paralyzed by venom-tipped needle. 5 x.

of the wasp must be done under reasonably high power of a dissecting microscope. The wasp itself can be held with the aid of watch-makers forceps or by a fine glass capillary connected to a suction hose. A very useful technique is the use of the forceps in one hand to hold the wasp and the suction capillary in the other to orient the insect in the proper position for applying the venom to the tip of the needle (Figure 7).

The blood itself from a paralyzed larva carries the venom in solution and serves as the simplest source of venom, and transfer of this blood offers a ready means of inducing paralysis in other larvae.

EFFECTIVE ROUTE OF ADMINISTRATION

Venom-containing blood from paralyzed larvae injected enterally into healthy *Galleria* larvae fails to induce paralysis, thus indicating that this route of administration is ineffective. Either the gut contents or gut tissues detoxify or dilute the venom to the point of its being innocuous, or the venom fails to penetrate the gut wall. Similarly, fluids containing the venom are ineffective when applied topically. Apparently the venom cannot penetrate the integument—at least the intact integument. In attempts to induce stinging by holding the wasp against a host larva in an appropriate position, it has been observed that the larvae become paralyzed even though the stylets show no apparent penetration of the integument and no apparent venom is ejected. The stylets are so small and so flexible that unless the wasp itself moves them as it normally does when stinging, they cannot be forced through the tough integument of a *Galleria* larva. In the cases just mentioned, it is believed that the sharp tip of the stylets in rubbing against the integument causes minute abrasions which could permit the penetration of the venom. The potency of the venom is so great that extremely small amounts, seeping out against the abraded surface, could easily escape detection and still be effective. For all practical considerations it may be concluded that injection into the body cavity is the only effective route of administration.

POTENCY OF THE VENOM

High potency of the venom used by *Habrobracon* in inactivating its prey is suggested by the prompt paralysis induced, from which there is no recovery. It is emphasized by the size differential of the two insects; a *Galleria* larva may be 1,000, or more, times the size (by weight) of a *Habrobracon* adult (Figure 8). The efficiency of the wasp and the potency of the venom in killing numbers of *Galleria* larvae, as mentioned above and illustrated in Figure 5, appear especially striking when it is considered that the wax moth larva is not readily killed by most of our common insecticides. Although the larvae are reasonably sensitive to arsenic, they have been known to survive immersion in pure alkaloid nicotine. They are only moderately susceptible to DDT, but are surprisingly tolerant of parathion, one of the most toxic and non-specific insecticides in common use.

Precise estimates of potency are impossible to make without special ultramicrotechniques which have not been available. There are no readily available means of determining the quantity of venom injected into a host larva during the stinging process. Considering the morphology of the poison

apparatus, it would seem reasonable that the maximum volume of venom injected at one time would be the volume of the poison reservoir, but this, too, is unknown. Ordinarily the wasps do not maintain the stinging position any great length of time. This suggests that a dose of venom is ejected at once and is not continuously pumped into the victim. As discussed above, female wasps can be induced to eject venom by holding them with forceps or with the tip of a capillary tube to which suction is applied. When the venom is discharged, it forms a minute droplet at the tip of the stylets. As far as can be judged from such a small droplet, it does not seem to enlarge as would be the case if the venom was being continually pumped out. If the droplet is removed, another droplet may be discharged after a longer or shorter interval. Unless secretion into the poison reservoir is very rapid, this indicates that the entire contents of the reservoir is not emitted at once. Although there is no assurance that the volume of a droplet formed at the tip of the stylets is of the same order of magnitude as that injected into an intended victim, measurement of such a droplet could serve as at least one

Figure 8. Female *Habrobracon* feeding on blood of *Galleria* larva. Note size difference between predator and host. 5 x.

approximation. The precision of this is open to question, but the basis of this technique is not entirely unreasonable. In the absence of ultramicropipettes suitable for volumetric work, measurement of a droplet of venom was made by assuming that the droplet as it hung from the tip of the stylets was a sphere, and noting its diameter through a microscope equipped with an ocular micrometer. It is difficult to make such measurements with accuracy and in numbers sufficient for statistical treatment, but this seems scarcely necessary. From the measurements made and converted to volumes for spheres, the quantity of venom discharged at one time appears to be from 20 to 65 micro-microliters (= 0.000000065 ml.). There can be no doubt that such a quantity can induce paralysis in a *Galleria* larva, but one cannot be sure that this is the range of magnitude of the venom naturally introduced

into the host when *Habrobracon* stings. In any case the volume is so small that an error of even a hundredfold would not be very serious.

As is discussed elsewhere, the venom is injected into the body cavity where it comes in contact with and is taken up by the blood and promptly circulated. Whether the entire quantity of toxin is held in solution in the blood, the chemical acting as a catalyst, or whether the chemical enters into a reaction, at its site of action, which removes it from circulation cannot be stated with finality. This will be discussed further in a later section.

The blood volume of *Galleria* larvae has been estimated with reasonably satisfactory techniques to be about 40 volumes per cent body weight (Richardson *et al.*, 1931). A working average weight of *Galleria* larvae may be considered to be 190 milligrams. This represents an average weight of 300 reasonably large larvae used for experimental purposes. Weights may be in excess of this, and smaller larvae may be used. This figure means that a blood volume of some 76 microliters might be expected from a moderately large larva. Considering the volume of venom injected, as estimated above, the venom concentration in the blood of a paralyzed larva would be something less than one part per million.

It has been mentioned that blood from a paralyzed larva can effectively induce paralysis when injected into a second larva. Moreover, blood from this larva can induce paralysis in a third larva. With small larvae this successive transfer can be carried to the fourth larva, but this is unusual. In view of the blood volume of the larva, considerable dilution is thus represented. By actually weighing the experimental larvae and calculating the blood volumes involved in the successive transfers, it was estimated that the presence of one part of venom in 200,000,000 parts of host blood was sufficient to cause a permanent paralysis. Lesser quantities can cause temporary paralysis. Such an estimate is, of course, based on the assumptions that have been discussed.

The first observations that the venom can be diluted and still be effective were made by the technique of transferring blood from paralyzed to healthy larvae. These observations raised the question as to whether the venom is effective after being greatly diluted or whether the venom itself is self-propagated in the host in a virus-like manner.

Several observations indicate the improbability of any self-propagation of the venom. For one thing, except with small larvae or in unusual cases, more than two successive transfers of poisoned blood from larva to larva are not effective even though sufficient time is allowed for a build-up of the venom if such existed.

Larvae paralyzed by the injection of low concentrations of venom recover after a period of time, the time being a function of the concentration.

If the venom was self-propagating, the potency of venom-containing blood should increase with time. In general a decrease in potency is noted with increased time after the stinging occurs. The decrease, however, is not so great as might be expected, as can be seen from the following data, which represent the responses of *Galleria* larvae to serially diluted venom-blood taken on successive days from larvae paralyzed at the beginning of the test.

Per Cent Paralysis[1] of *Galleria* Larvae

Dilution of venom-blood	Days after paralysis of larvae supplying venom-blood				
	0	1	2	3	4
1 - 5	97%	97%	83%	97%	80%
1 - 15	93	87	68	77	62
1 - 45	53	52	20	42	62
1 - 135	14	15	8	0	0
1 - 405	0	5	0	5	5

[1] For explanation of term "per cent paralysis", see text below.

The data just presented indicate a dosage-response, that is, a reduced degree of paralysis resulting from injections of more diluted venom. The per cent paralysis recorded is based on a scoring system in which dead larvae or larvae completely paralyzed are scored "3"; larvae incapacitated but showing some slight movement, either spontaneous or in response to tactile stimulation, are scored "2"; larvae showing uncoordinated movement, but which are able to show locomotor activity, are scored "1"; unaffected larvae receive no score. A maximum score is $3n$, where n is the number of larvae in the test group, and percentage paralysis of the group is based on this value. Data of this kind are satisfactory only for direct comparisons made at uniform times. They suggest the degree of paralysis at the particular time of observation, but the picture changes from the time of administration of the venom until a stabilized end-point is reached at which time all of those that are able to do so recover and all others are in a state of irreversible paralysis or death. The changing picture is schematically illustrated in Figure 9. High concentrations cause a prompt paralysis from which there is

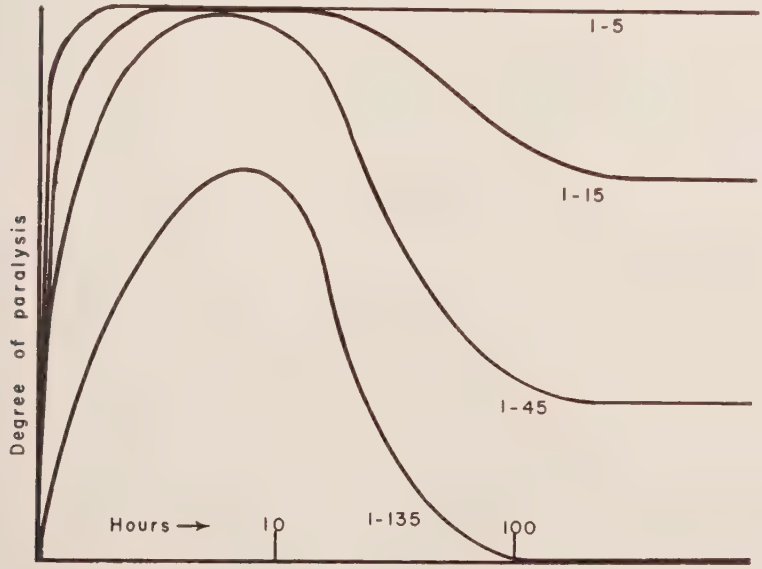

Figure 9. Chart showing, schematically, the relationships between venom concentration and onset of paralysis, degree of paralysis, and time of recovery.

no recovery. Lower concentrations result in delayed paralysis, and the more dilute the venom, the fewer larvae there are which become paralyzed, and the more rapid is their recovery.

The female *Habrobracon* as a "poison factory" can be appreciated if one indulges in some arithmetic speculation. Considering the possibility of successive blood transfers resulting in complete paralysis followed by death, and if one has at hand a large group of *Galleria* larvae, each of which weighs 100 milligrams and contains 40 microliters of blood, the following numbers of larvae could be paralyzed if one microliter of blood served as the inoculum and all the blood could be utilized:

Originally stung larva	1
Primary inoculations	40
Secondary inoculations	1600
	1641

A single female wasp, however, can sting many larvae. The number stung depends largely on the female's longevity as it can sting only a few each day. As many as 175 *Galleria* larvae have been observed to be stung by one female. If, then, each of these 175 larvae could have been treated as above, this single female, produced, speculatively, sufficient poison to kill 227,175 larvae, or about 50 pounds of larvae.

In the course of studies involving dilution tests of venoms, it has been apparent —as may be evident from the different tables throughout this paper —that the potency of venom-containing blood varies rather broadly. This could suggest that at different times different quantities of venom are injected into hosts, that is, the initial concentration varies from sample to sample. Although this is very possible, the fact that pooled samples are used would tend to minimize this effect. Another possibility is that the actual potency varies—either as a result of differences in venom secretion or in antagonistic mechanisms which might be operating in the host. Periodic assays were not made in a uniform manner, so the range of variation in the venom-containing bloods is not known.

SITE OF ACTION OF THE VENOM

The ligature tests mentioned previously demonstrate that segmental tissues rather than some single, localized organ are affected by the venom to result in loss of muscle tone. Thus, the segmental ganglia, the nerve connectives, the motor nerves to the muscles, the neuromuscular junction, or the musculature itself would be suspected sites of action. The fact that the heart and alimentary tract are apparently unaffected need not be surprising in view of the separate innervations these organs receive and the differences in muscle structure as compared with the body wall musculature.

It has generally been assumed that the venoms of predaceous wasps are neurotoxic, and Hartzell (1935) presented histopathological evidence to the effect that ganglia in the cicada (*Tibicen pruinosa* Say) paralyzed by the sting of *Sphecius speciosus* Dru. showed characteristic lesions which were similar in appearance to those observed in the same insect paralyzed by pyrethrum. Richards and Cutcomp (1945) criticized this interpretation by observing that such histological changes can well be a consequence of autolysis associated with a general degenerative condition.

and do not necessarily indicate a specific action of the toxicant on the ganglion nor reflect a causal mechanism of action of the chemical.

At present, pharmacological techniques for studying neuromuscular function in insects are not generally satisfactory because the diagnostic agents so useful in vertebrate studies do not have the same effects in insects, and comparable drugs effective in insects are not known with the same degree of assurance.

Electrophysiological tests are more informative. The techniques such as described by Roeder (1952) are applicable to the neuromuscular system of *Galleria* larvae. The apparatus used in these studies included finely tapered silver electrodes for recording and stimulating. The recording electrodes were led through a pre-amplifier to an oscillograph and loudspeaker. The stimulator was a rectangular wave type generator, in which the stimulus could be varied in frequency, intensity, and duration.

Considering the flaccid paralysis of the *Habrobracon* victims, it is not surprising that electrodes placed in the body wall musculature do not pick up spontaneous potentials such as are evident in normal larvae as characteristic muscle action potentials (Figure 11). Such absence of muscle

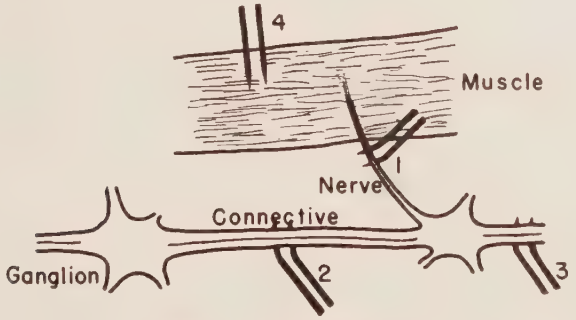

Figure 10. Diagram of neuromuscular structures and electrode positions for determining functional activity.

activity could result from direct action on the muscles themselves or on some portion of the segmental nervous system by means of which impulses are carried to the muscle to maintain body tonus and permit normal activity.

If recording electrodes are placed on the interganglionic connectives (position 2, 3, Figure 10) of a normal larva, volleys of spike potentials characteristic of nerve activity are seen on the oscilloscope (Figure 11). If stimulating electrodes are placed on one side of a ganglion (position 2) and recording electrodes on the other (position 3), the response to a single electrical stimulus is obscured by the spontaneous activity. Mechanical or chemical stimuli, on the other hand, increase the frequency and amplitude of the nerve spike potentials and indicate ganglionic transmission of the nerve impulses. On the other hand, if the ganglion is injured mechanically or chemically, the spontaneous activity ceases. In the paralyzed larva these

same phenomena can be observed, and it may be concluded that paralysis is not induced by injury to the ganglia nor to the connectives. This at once is contrary to the opinion based on Hartzell's observations (on different species, to be sure), which attribute the pathology to injured ganglia.

If recording electrodes are placed on the nerves leading from the ganglia to the body wall musculature (position 1, Figure 10), spontaneous nerve potentials are evident both in normal and paralyzed larvae. If these nerves are electrically stimulated, the muscles in the normal larva respond by

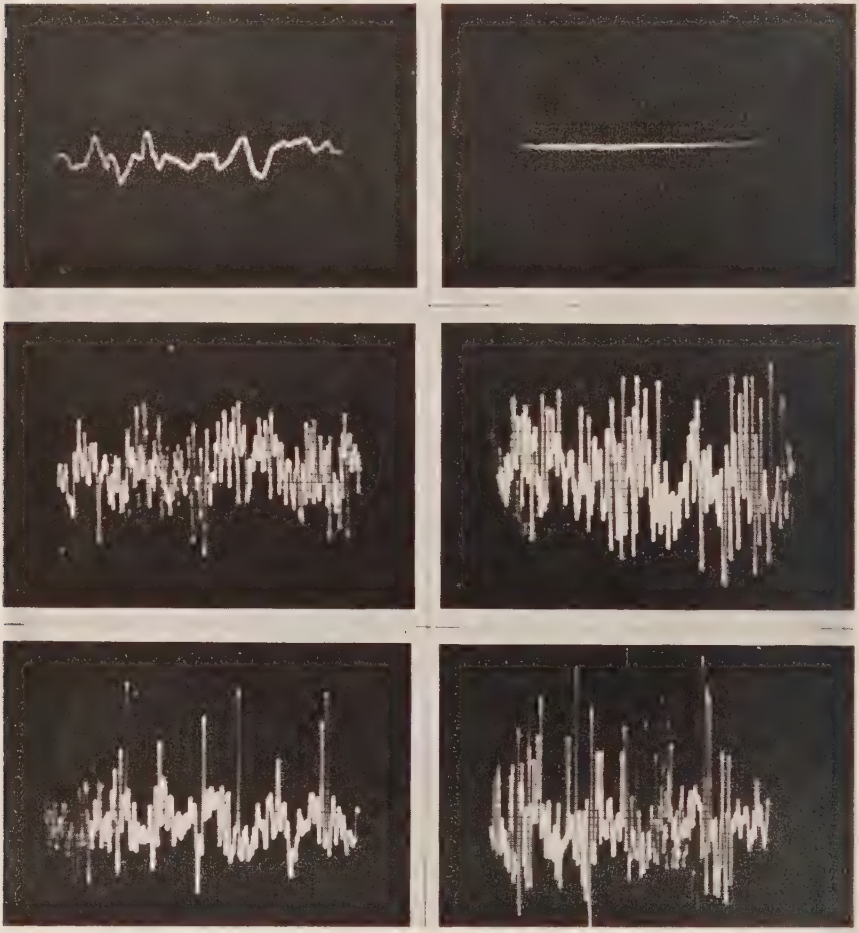

Figure 11. Oscillograph records of muscle and nerve action potentials. Upper left, trace showing characteristic muscle action potentials of normal *Galleria* larva. Upper right, trace showing absence of muscle action potentials of paralyzed larva. Center, traces showing nerve action potentials of normal larva; spontaneous on left, following mechanical stimulation on right. Lower, traces showing nerve action potentials of paralyzed larva; spontaneous on left, following mechanical stimulation on right.

vigorously contracting even with stimuli of low intensity. On the other hand, stimulation of these nerves in a paralyzed larva fails to elicit a muscular response.

If no response can be elicited by the stimulation of motor nerves, and all nerves show spontaneous activity, there is little need to consider the possibility that sensory nerves or their endings are responsible for the paralysis.

Direct electrical stimulation of the muscles in a paralyzed larva fails to cause contraction except at unusually high voltages. This reduced response to electrical stimulation is not strictly coincident with the appearance of the flaccid condition, but after paralysis, increasing voltages are required to elicit any response, until ultimately there is negligible response to the highest voltage applied (70 volts). Injection of some few drugs such as nicotine or hyoscyamine cause paralyzed larvae to draw up and lose their flaccid appearance. But whether this reaction and the response (though weak) to high voltage stimulation indicate that irritability rather than contractility of the muscles is affected is not clear. Interpretation largely hinges on the response of normal muscle alone to electrical stimulation. The body wall musculature of normal *Galleria* larvae seems rather fragile and becomes unresponsive under several conditions. Although the muscles contract readily when very low voltages are applied to nerves supplying them, they do not respond to direct stimulation when (in a dissected state) the nerves are removed. Healthy larvae under carbon dioxide anaesthesia are unresponsive to direct muscle stimulation. Larvae narcotized with nicotine do respond to muscle stimulation, but require stimuli of greater intensity than normal. This latter observation may be reconciled with the other observations because nicotine acts primarily on ganglia and secondarily on peripheral tissues. It seems that the *Galleria* body wall muscles are especially sensitive to disturbances that affect their irritability. This is less true of *Ephestia* larvae, for when paralyzed by *Habrobracon*, their muscles are responsive to direct electrical stimulation. If it is assumed, as is reasonable, that both species of larvae are affected alike, this offers the most convincing evidence that the contractility of the muscles is not impaired. This, then, suggests that the excitation process of the muscles is impaired and places the most probable site of action at the neuromuscular junction.

MODE OF ACTION OF THE VENOM

In seeking information on the mode of action, the physiological approach involves the testing of chemical compounds known to have some action on one biological system or another in an effort to find any that simulate the venom when applied to normal larvae, alleviate the paralytic symptoms in the stung larvae, or that antagonize or augment the action of the venom. Such a physiological approach can only be a first approximation and cannot be substituted for biochemical methods which ultimately will have to be relied upon to give a final answer. The small quantity of venom available precludes the use of some of the classical techniques which could be very informative. The sensitivity of the physiological response, however, is a great advantage in this approach.

Galleria larvae exhibiting DDT tremors are readily quieted with *Habrobracon* venom. This would be expected in view of the fact that the DDT

tremors result from disturbances in the sensory nerve endings whereas muscle excitation is impaired by the venom. Thus, even though the stimuli are sent into the nervous system, their manifestation as muscle tremors is blocked. It is somewhat surprising to note, however, that DDT-poisoned larvae are avoided by *Habrobracon* females. Probably the abnormal movement of the host discourages them, rather than there being any perception on their part of a situation that would be deleterious to themselves or their offspring.

Histamine has been identified in bee venom, and much of the irritation in humans resulting from bee and wasp stings is attributed to this chemical. Histamine diphosphate, injected into normal *Galleria* larvae, has no apparent effect. Moreover, the histamine antagonist pyribenzamine has no ameliorative action when injected into a larva paralyzed by *Habrobracon* venom.

Especial interest in recent years has been evident in choline-ester-esterase systems because of the antagonism for the enzyme exhibited by parathion and other organic phosphate insecticides. There has been increasing physiological evidence, however, to indicate that the chemical mediator in motor nerves of insects that triggers muscular action is not acetylcholine nor even related choline esters. In other words, the motor nerves to the skeletal muscles of insects are not cholinergic as they are in higher animals. Presumably, however, choline-ester-esterase systems are operating in insects, but not at the neuromuscular junction. Physostigmine is a drug highly effective in inhibiting cholinesterase. In low concentrations it produces a reversible paralysis in *Galleria* larvae. If *Habrobracon* venom acted upon the same system as physostigmine, it would be expected that physostigmine, in non-lethal concentrations, would augment the action of *Habrobracon* venom. This was tested by injecting physostigmine (three microliters of 10^{-5} M solution) into each larva of a group. The larvae were in turn injected with venom-containing blood in a dosage series, along with a parallel control series of larvae not receiving the physostigmine. The degree of paralysis, based on the scoring system previously mentioned, was noted four days after treatment as follows:

Dilution of venom-blood	Per cent paralysis	
	Venom-blood alone	Venom-blood plus physostigmine
1 - 5	100%	100%
1 - 15	97	93
1 - 45	83	81
1 - 135	33	43
1 - 405	21	23

Obviously the physostigmine did not augment the effects of the venom, and if one can judge by this technique, the venom can be said to act in some other way than by inhibiting cholinesterase.

An enzyme system very important in insect muscle metabolism is the cytochrome system. This system is affected by DDT (Sacktor, 1949) and the synthesis of one or more of its components in insects is blocked by diphtheria toxin (Pappenheimer and Williams, 1952). Sodium azide and cyanide are well known inhibitors of the cytochrome oxidase system, and if *Habrobracon* venom acts similarly, these compounds should augment the

paralytic action. Sodium azide was tested in the same way as physostigmine mentioned above. Injections of three microliters of sodium azide (.04 M) prior to injections of venom-blood yielded the following results:

Dilution of venom-blood	Per cent paralysis: Venom-blood alone	Venom-blood plus sodium azide
1 - 4	87%	83%
1 - 8	80	70
1 - 16	63	70
1 - 32	40	50
1 - 64	17	13

With no augmentation evident, it is doubtful if the venom has any effect on the cytochrome oxidase. Further evidence that the venom does not act on the cytochrome system lies in the probability that cytochrome activity is to be found in heart and gut as well as in the body muscle, as found in the cockroach (Sacktor and Bodenstein, 1952), although it is absent or low in the pupal heart of the giant silkworm, *Cecropia* (Pappenheimer and Williams, 1952). General inhibition of the cytochrome system should affect the heart and gut, then, as well as the body muscles —which is not the case in paralyzed larvae. Also, a general inhibitor of the cytochrome system should be less species-specific than is *Habrobracon* venom.

It has been found by Edwards *et al.* (1948) that the insecticide ryania, through its active principle ryanodine, exerts its effect by interfering with the contractile processes in the body muscles. Although it seems evident that *Habrobracon* venom interferes with muscle excitation rather than with contraction, comparison with ryania might help confirm or deny a difference. Aqueous extracts of ryania injected into *Galleria* larvae induce a paralysis from which they are likely to recover. Loss of tone is not so severe as with venom. European corn borer (*Pyrausta nubilalis* (Hbn.)) larvae are more susceptible to ryania than are *Galleria* larvae, but do not become paralyzed by *Habrobracon* venom. Although these differences could arise as a result of different cellular permeation effects, they are more likely because of true differences in mode of action, and it appears probable that the chemicals act on different systems.

In an empirical approach, numerous compounds known to be active on one biological system or another were tested on normal larvae to see if the venom-paralysis could be simulated, or on the other hand, if the paralysis in stung larvae could be relieved. Most compounds were ineffective in both regards. A few, however, resulted in toxic symptoms more similar to the venom-induced paralysis than those caused by most other toxicants. These seemed to have in common the ability to complex metals. Upon further testing they were found to be required in too high a concentration or were too inconsistent in their action to be considered as having real physiological significance. Other chelating agents were tested, and of the entire group (which was not exhaustive by any means) three proved to be of more than passing interest. These were tetrasodium ethylenediamine tetraacetate, sodium dimethyl dithiocarbamate, and 1, 10 phenanthroline. Of these, the sodium dimethyl dithiocarbamate was the most potent, causing reversible paralysis when injected in amounts of three microliters per larva of solutions somewhat less concentrated than 0.25 per cent, and death at higher concentrations. The paralysis induced by these compounds was not identical

symptomatically with that induced by *Habrobracon* venom. The flaccidity was less complete and the heart action was decreased, if not stopped. Although the evidence, by analogy, that the venom might act as a chelating agent was not very convincing, it suggested the testing of metals as possible antagonists of the venom. Accordingly, salts of strontium, nickel, cobalt, iron (ferrous and ferric), copper (cuprous and cupric), zinc, manganese, and magnesium were tested in a preliminary way as antagonists of the venom by mixing them with venom-containing blood and injecting the mixture into *Galleria* larvae. Characteristic paralysis occurred in all cases except where cupric salts were used. Further testing indicated that copper sulfate, for instance, is toxic to larvae in its own right, but if different concentrations are added to aliquots of blood containing uniform concentrations of venom and injected into healthy larvae, a complex response is evident. At high concentrations of copper the larvae are killed by the chemical, and the effect of the venom (if any) is masked. As the concentration of copper is reduced, its toxic effect is reduced, and its antagonism to the venom becomes evident. At concentrations of about .005 M it is relatively non-toxic to larvae, but inhibition of the venom activity is still almost complete and the treated larvae are scarcely affected by either the copper or the venom. As the concentration of copper is reduced still further, antagonism is less complete and the treated larvae show increased paralysis because of the venom. It was found, however, that copper injections failed to alleviate the paralysis of stung larvae, and copper injections prior to stinging or other administration of the venom failed to protect the larvae from paralytic action.

In one test, sub-lethal concentrations of tetrasodium ethylenediamine tetraacetate showed some tendency to augment the action of the venom.

These observations on chelating compounds and copper antagonism are not sufficient to warrant much speculation on the venom acting as a chelating compound, but they are of sufficient interest to warrant further investigation in this direction.

Certain comparisons and contrasts may help to orient thought on the mode of action of the venom.

In the symptoms caused, in the effective route of administration, and in the site of action, the venom-effect in *Galleria* most resembles the curare-effect in vertebrates. The poisoned arrow analogy mentioned in the introduction is especially fitting. However, curare is not effective in insects, so their actions cannot be identical.

The potency of the venom recalls the extreme potency of the bacterial toxins. As mentioned above, Pappenheimer and Williams (1952) found that diphtheria toxin interferes with muscle metabolism of insects by blocking the synthesis of one or more components of the cytochrome system. Although the venom certainly does not act in this way, it is interesting to note that whereas diphtheria toxin is antagonized by iron, *Habrobracon* venom is antagonized by copper.

Although at this stage of the investigation it would be pure speculation to postulate that *Habrobracon* venom paralyzes *Galleria* larvae by chelating the copper of some, at present, unknown copper-containing mediator of the nerve impulse in muscle tissue, this would be the best guess that the fragmentary evidence permits.

THE NATURE OF *Habrobracon* VENOM

It will be difficult to learn much about the chemistry of the venom because of the extremely small volumes obtainable. That it is a protein or protein-like material is suggested by the fact that it is heat-labile. At a temperature of about 65° C. or higher, the venom is inactivated. Blood containing venom can be safely heated to prevent coagulation at temperatures of 55 to 60° C. with no loss of potency. Although the technique might be questioned because of the small volumes used, the venom was found not to pass through a dialyzing membrane. The venom is water-soluble and can stand desiccation. Dried venom can be taken up in water and used to paralyze larvae. Washed cells from centrifuged blood from stung larvae do not induce paralysis when injected into healthy larvae, but the supernatent is effective. Thus, the venom is to be found in the plasma portion of the blood. Many of the blood proteins may be precipitated with low concentrations of alcohol without affecting the potency of the contained venom. Phenylthiourea does not affect the potency of the venom, so this may be used in preventing the melanization of the venom-containing blood for use in paralyzing larvae.

In view of the probably high molecular weight of the venom and its high potency, relatively few molecules must be required at the site of action. Also, considering the relatively slow loss of titer of venom-containing blood, it might be conjectured that it acts as a catalyst and may itself be an enzyme.

SPECIES SPECIFICITY OF *Habrobracon* VENOM

In the sense that species-specificity is relative and not absolute, *Habrobracon* venom may be said to be quite species-specific. Relatively few insect species have been tested, but sufficient have been treated with the venom to indicate that susceptibilities vary from extreme sensitivity to complete resistance. If a large number of species was tested, the results would probably show a preponderance of species resistant to the venom, the others showing a complete range of response.

Among the species tested, *Galleria*, *Ephestia*, and *Plodia* larvae are, as mentioned before, very susceptible, and wasps do not hesitate to attack them. Paralysis is complete and there is no recovery. At the other extreme, larvae of the Japanese beetle (*Popillia japonica* Newm.), European corn borer (*Pyrausta nubilalis* Hbn.), and nymphs and adults of the large milkweed bug (*Oncopeltus fasciatus* (Dall.)) are not attacked by wasps, and injections of venom fail to cause any apparent responses other than those that might result from the injection technique alone. Wasps will voluntarily sting Oriental fruit moth larvae (*Grapholitha molesta* (Busck)), although in one test they failed to oviposit on them. A definitely intermediate reaction was noted in larvae of the ugly-nest cherry worm (*Archips cerasivorana* (Fitch)). These larvae were stung by the wasps, but very reluctantly, and paralysis resulted. In larvae stuck with "poisoned arrows", paralysis finally ensued, but onset was delayed much longer than in *Galleria*. Poisoned larvae were very sensitive to mechanical stimulation after cessation of locomotor activity, giving the impression that paralysis was not going to be complete. Ultimately, however, paralysis with complete loss of muscle tone resulted. Among the few *Archips* larvae observed, there was

no recovery, but in view of the time required for full paralysis to occur, it would not be surprising to find some larvae recovering after exposure.

The question arises as to why some insects are susceptible to the venom and others are not. Is resistance attributable to lack of a chemical substrate sensitive to the venom or is it associated with defensive mechanisms which antagonize the venom or prevent the venom from reaching the site of action? These questions are amenable to experiment, using the European corn borer larva as a resistant form. The insensitivity of corn borer larva to this venom is especially surprising in view of the fact that the insect is very commonly parasitized by a closely related species of *Habrobracon*, namely, *H. brevicornis*.

If a "poisoned arrow" is introduced into the body cavity of a corn borer larva, no paralysis is induced —thus demonstrating its resistance. That the "arrow" is indeed poisoned can be demonstrated by inserting the same needle into a *Galleria* larva. Even after being in a *Pyrausta* larva, the needle still is effective in causing paralysis in *Galleria*. If the venom is not taken up by the blood of *Pyrausta*, or if the blood antagonizes the venom, this should become evident if blood from such a treated *Pyrausta* larva is injected into *Galleria* larvae. If this is done, characteristic paralysis of the latter results, indicating that *Pyrausta* blood does take up the venom and circulates it, but does not antagonize it. The absence of antagonism of the venom by *Pyrausta* blood is further demonstrated by using *Pyrausta* blood as a diluent in comparison with water. In such a test, the venom is serially diluted in one series with *Pyrausta* blood and in a companion series with water, each test solution being injected into groups of *Galleria* larvae. The results, noted two days after injection, are expressed as follows:

	Per cent paralysis	
Dilution	Diluent: *Pyrausta* blood	Water
1 - 5	100%	100%
1 - 20	96	96
1 - 80	93	87
1 - 320	53	48
1 - 1280	37	7

It is obvious that, *in vitro*, *Pyrausta* blood in no way impairs the effectiveness of the venom. The tendency for the *Pyrausta* blood-diluted venom-blood to be slightly more effective is probably without significance.

From the above discussion it should be apparent that in considering host specificity of *Habrobracon*, distinction should be made between selection of hosts by the wasps in their stinging activities, the ability of hosts to invite oviposition and to nourish the *Habrobracon* offspring, and the species-specificity of the venom itself as a toxicant.

Host preferences by *Habrobracon* are evident even among those insects which it readily attacks. If *Galleria* and *Ephestia* larvae are both present, *Ephestia* are the first to be stung and fed upon. This holds true even though the wasps were reared on *Galleria* and for several generations. Certainly Hopkin's "host selection principle" has no application to this host-predator relationship.

Also, *Ephestia* larvae are superior to *Galleria* larvae for *Habrobracon* reproduction. Eggs are deposited sooner on *Ephestia* than on *Galleria*, and

in spite of their smaller size they seem to support larger numbers of *Habrobracon* larvae.

The specificities exhibited by response to the venom indicate subtle biochemical differences. There must be slight differences in the chemical mediators of the nerve impulse among species and there are likely to be appropriate differences, too, among the venoms of the different parasitic and predaceous wasps.

THE PARALYZED LARVA AS A PREPARATION FOR PHYSIOLOGICAL STUDY

The *Galleria* larva paralyzed by *Habrobracon* offers exceptional possibilities as an experimental animal for certain physiological studies.

The heartbeat without the interference of other muscle activity can be studied on the relatively intact animal. Action potentials of the heartbeat can be very simply obtained without dissection by inserting electrodes through the body wall and in contact with the heart. The rhythmic trace can be observed on the oscilloscope or suitable recording devices could be used. In view of the fact that the animal is intact except for the punctures made by the electrodes—and these readily seal over—, chemical substances can be injected into the larva and the action on the heart observed. Quantitative expression of final blood concentration is permitted by calculation from the estimated blood volume.

Stung larvae can serve as permanently anaesthetized subjects for study of the pharmacology of the central nervous system as approached through electrophysiological techniques.

If the site of action has been correctly determined, *Habrobracon* venom can serve the same very important pharmacological tool for studying *Galleria's* neuromuscular physiology that curare serves for vertebrates. This can be an important step in learning more about the excitation process in insect muscle.

SUMMARY

Habrobracon juglandis Ashmead is one of many predaceous wasps that subdue their victims by injecting into their bodies a paralyzing venom. The flaccid paralysis is irreversible, but until the host (*Galleria*) larva dies, the heart and gut muscles continue to function.

There is no evidence that the injected venom has any antiseptic qualities.

The wasp does not sting its host in any special locus, but blood transport assures the venom's reaching its site of action. Injection into the body cavity (blood stream) is the only effective route of administration.

The venom is extremely potent against *Galleria* larvae. Although estimates of potency require certain assumptions, the amount of venom introduced relative to the blood volume of the host approximates one part per million. It has been further estimated that dilution of the venom is possible to the extent that one part of venom to 200 million parts of host blood is sufficient to induce permanent paralysis. Variation in potency occurs, how-

ever, and these estimates may not be reached in some cases and may be exceeded in others.

The paralysis results from impairment of the excitatory processes of the body wall musculature, and the site of action is therefore considered to be the neuromuscular junction.

The mode of action of the venom is not known, but it is certainly different from any of the well-known insecticides. Certain speculations as to mode of action are made.

The effects of *Habrobracon* venom in *Galleria* larvae are remarkably similar to the curare effects in vertebrates.

Striking differences in susceptibility to the venom are evident among different species of insect. Lack of susceptibility is not a result of gross antagonistic mechanisms. It is more likely because of a slight difference in the substrate acted upon by the venom, cellular barriers at the site of action, or antagonisms at this level.

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